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BRIEF REPORT

CRISPR Correction of the *GBA* Mutation in Human-Induced Pluripotent Stem Cells Restores Normal Function to Gaucher Macrophages and Increases Their Susceptibility to *Mycobacterium tuberculosis*

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Gaucher disease (GD) is an autosomal recessive lysosomal storage disorder caused by mutations in the β-glucocerebrosidase (GCase) *GBA* gene, which result in macrophage dysfunction. CRISPR (clustered regularly interspaced short palindromic repeats) editing of the homozygous L444P (1448T→C) *GBA* mutation in type 2 GD ($GBA^{-/-}$) human-induced pluripotent stem cells (hiPSCs) yielded both heterozygous ($GBA^{+/-}$) and homozygous ($GBA^{+/+}$) isogenic lines. Macrophages derived from $GBA^{-/-}$, $GBA^{+/-}$ and $GBA^{+/+}$ hiPSCs showed that *GBA* mutation correction restores normal macrophage functions: GCase activity, motility, and phagocytosis. Furthermore, infection of $GBA^{-/-}$, $GBA^{+/-}$ and $GBA^{+/+}$ macrophages with the *Mycobacterium tuberculosis* H37Rv strain showed that impaired mobility and phagocytic activity were correlated with reduced levels of bacterial engulfment and replication suggesting that GD may be protective against tuberculosis.

Keywords. CRISPR/Cas9 editing; Gaucher disease; human-induced pluripotent stem cells (hiPSCs); macrophage motility; *Mycobacterium tuberculosis* uptake and multiplication.

Human β-glucocerebrosidase (GCase) comprises 497 amino acids with 4 oligosaccharide chains coupled to specific asparagine residues [1]. GCase is a lysosomal hydrolase that breaks down its substrates, glucosylceramide (GluCer) and glucosylsphingosine (GluSph), into glucoseplus ceramide, and sphingosine, respectively. Its deficiency causes GluCer and GluSph to accumulate within lysosomes of Gaucher macrophages, resulting in lysosomal dysfunction. Gaucher disease (GD) is characterized by lipid-laden Gaucher macrophages that infiltrate the bone marrow and other visceral organs [2–4]. Three clinical forms of GD (types 1, 2, and 3) have been identified, of which type 2 GD is the most severe.

Since GD is a recessive disorder, the mutations occur in both alleles of the *GBA* gene in patients' cells. While the common N370S mutation is associated with type 1 GD, the severely destabilizing L444P (1448 T→C) mutation is strongly associated with types 2 and 3 GD. Elevated levels of inflammatory mediators (tumor necrosis factor [TNF] α, interleukin 6 [IL-6], and interleukin 1β [IL-1β]) have been reported in the serum of patients with GD [3, 4], and GD macrophages have been shown to have migratory defects [5, 6]. Type 1 GD, which occurs at high frequency in Ashkenazi Jews (carrier rate, approximately 1 in 15), is thought to have originated 800–1400 years ago [7, 8], and the persistence of this and other types of GD mutations in human populations at relatively high levels has prompted the concept that GD mutations may confer a selective advantage. One theory holds that GD homozygosity and/or heterozygosity may be protective against common lethal human infections such as tuberculosis [9, 10].

METHODS

CRISPR Correction of *GBA* Mutation in GD Human-Induced Pluripotent Stem Cells

Type 2 GD primary fibroblasts without any patient identifiers (catalogue no. GM08760) were purchased from the National Institute of General Medical Sciences repository at the Coriell Institute. We previously published a detailed report on the generation of human-induced pluripotent stem cells (hiPSCs) from type 2 GD (homozygous for GCase [*GBA*] 1448T→C mutation in exon 10 which results in the L444P amino acid substitution) patient fibroblasts using CCR5-specific TALENs (transcription activator–like effector nucleases) [11]. CRISPR (clustered regularly interspaced short palindromic repeats)–mediated gene correction was performed using monoallelic CCR5-modified GD hiPSCs, using methods described in the Supplementary Materials [11].

Macrophage Functional Assays

Macrophage GCase enzymatic assays, phagocytosis activity, and motility were measured as described in the Supplementary Materials.

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Macrophage Infection Assay Using *Mycobacterium tuberculosis*

All macrophage infection experiments were performed in a Johns Hopkins University biosafety level 3 facility. Macrophage infections were carried out in 24-well plates in duplicate. For infection, early log-phase cultures of *Mycobacterium tuberculosis* strain H37Rv were washed and diluted appropriately to predefined concentrations in antibiotic-free Roswell Park Memorial Institute medium and were added to the isogenic $GBA^{-/-}$ $GBA^{+/-}$ and $GBA^{+/+}$ macrophages at a multiplicity of infection of 5:1. The infection was allowed to continue for 4 hours, after which extracellular bacteria were removed by washing the infected cells thoroughly with Dulbecco's phosphate-buffered saline. Serial dilutions of the bacterial suspension and macrophage lysate were plated at day 0 to determine an accurate bacterial count of infection and phagocytized bacterial number. For enumeration of bacterial growth at 1, 2 and 3 days after infection, cells were harvested and lysed using 0.025% sodium dodecyl sulfate. Appropriate dilutions of the lysates were then inoculated onto Middlebrook 7H11 agar plates in duplicate and incubated at 37°C for 3 weeks. The number of colonies was counted and expressed as $\log_{10}$ colony-forming units (CFUs) per well. Data are representative of 2 experiments. Infection experiments were conducted with and without macrophage activation by IFN-γ.

RESULTS

We had previously reported TALEN-mediated generation of type 2 homozygous L444P (1448 T→C) GD hiPSCs [11]. CRISPR/Cas9 (CRISPR-associated protein 9) editing of L444P mutations in GD ($GBA^{-/-}$) hiPSCs using a single-guide RNA and a 100–base pair single-strand oligonucleotide as the donor template yielded isogenic lines with heterozygous ($GBA^{+/-}$) and homozygous ($GBA^{+/+}$) gene correction (Figure 1*A* and Supplementary Figure 1). Since the L444P mutation results in an *Nci* I restriction site, we screened for $GBA^{+/-}$ and $GBA^{+/+}$ single-cell colonies by digesting an approximately 800–base pair polymerase chain reaction–amplified fragment surrounding the mutant locus with *Nci* I (Figure 1*B*). Sequencing of the polymerase chain reaction–amplified DNA further confirmed the genotypes of the $GBA^{-/-}$, $GBA^{+/-}$, and $GBA^{+/+}$ hiPSCs (Figure 1*C*). Expression of pluripotency markers in $GBA^{+/-}$ and $GBA^{+/+}$ hiPSCs was confirmed by immunostaining using antibodies for Oct4, Sox2, Tra-1-60, Tra-1-81, and DAPI (4′,6-diamidino-2-phenylindole), respectively (Supplementary Figure 2). Karyotyping of the $GBA^{+/-}$ and $GBA^{+/+}$ hiPSCs established that the cells were all normal (46XY) without any chromosomal abnormalities (Supplementary Figure 3) like the GD hiPSCs that were characterized previously [11]. Sequencing of 8 sites closely related to the single-guide RNA target in the $GBA^{+/-}$ and $GBA^{+/+}$ hiPSCs did not reveal any indels that were induced by non-homologous end joining, ruling out off-target cleavage by CRISPR/Cas9 (Supplementary Tables 1 and 2).

We differentiated the $GBA^{-/-}$, $GBA^{+/-}$, and $GBA^{+/+}$ isogenic hiPSCs, first into monocytes, and then into macrophages, using standard protocols (Figure 1*A* and Supplementary Figure 4) [3]. Western blot analysis using a GCase antibody confirmed that GCase expression is partially restored in $GBA^{+/-}$ and fully restored in $GBA^{+/+}$ macrophages (Figure 1*D*). We also monitored the GCase activity directly in $GBA^{-/-}$, $GBA^{+/-}$, and $GBA^{+/+}$ macrophages using 4-methylumbelliferyl-β-D-glucosylpyranoside as the substrate (Figure 1*E*). The results confirmed partial restoration of GCase activity in $GBA^{+/-}$ and full restoration in $GBA^{+/+}$ macrophages, compared with $GBA^{-/-}$ macrophages.

To determine whether Gaucher macrophages show abnormal chemokine activation and whether their functional defects could be reversed by L444P mutation correction, we examined the expression profiles of TNF-α, IL-6, IL-1β, and interleukin 10 (IL-10) in isogenic $GBA^{-/-}$ $GBA^{+/-}$, and $GBA^{+/+}$ macrophages (Figure 2*A*). Significant higher levels of IL-1β expression were observed in homozygous $GBA^{-/-}$ and heterozygous $GBA^{+/-}$ macrophages compared with $GBA^{+/+}$ *GBA*-corrected macrophages. IL-1β is an important inflammatory mediator that is involved in cell proliferation, differentiation, and apoptosis. Increased risk of cancers, autoimmune disease, and infections associated with human GD may be attributable in part to the elevated production of IL-1β by Gaucher macrophages. As with IL-1β, expression of the proinflammatory cytokines TNF-α and IL-6 trended towards being higher in isogenic Gaucher macrophages, while anti-inflammatory IL-10 appeared to be unaffected by *GBA* mutations. Our observations are consistent with previous studies reporting elevated TNF-α, IL-6, and IL-1β expression in Gaucher macrophages differentiated from patient-derived hiPSCs with types 1, 2, and 3 GD compared with control cells [2, 3].

Next, we examined whether CRISPR editing of the L444P mutation in GD hiPSCs restores normal phagocytic function and motility to Gaucher macrophages. We evaluated phagocytic functionality of GD ($GBA^{-/-}$), $GBA^{+/-}$, and $GBA^{+/+}$ macrophages by zymosan particle engulfment assay (Figure 2*B*); opsonization was not used in order to assess native phagocytosis capability independent of Fc receptor–mediated endocytosis. As expected, GD macrophages ($GBA^{-/-}$) showed only minimal engulfment of zymosan particles, while phagocytosis was moderately recovered in $GBA^{+/-}$ macrophages. In contrast, biallelic *GBA*-corrected macrophages ($GBA^{+/+}$) exhibited maximal engulfment of zymosan particles indicating that the L444P mutation correction restores normal phagocytic potential to Gaucher macrophages.

Since migration is critical for macrophage immune function, we compared the mobility of $GBA^{-/-}$, $GBA^{+/-}$, and $GBA^{+/+}$ macrophages by transwell migration assay using zymosan, a commonly used macrophage chemoattractant (Figure 2*C*). As

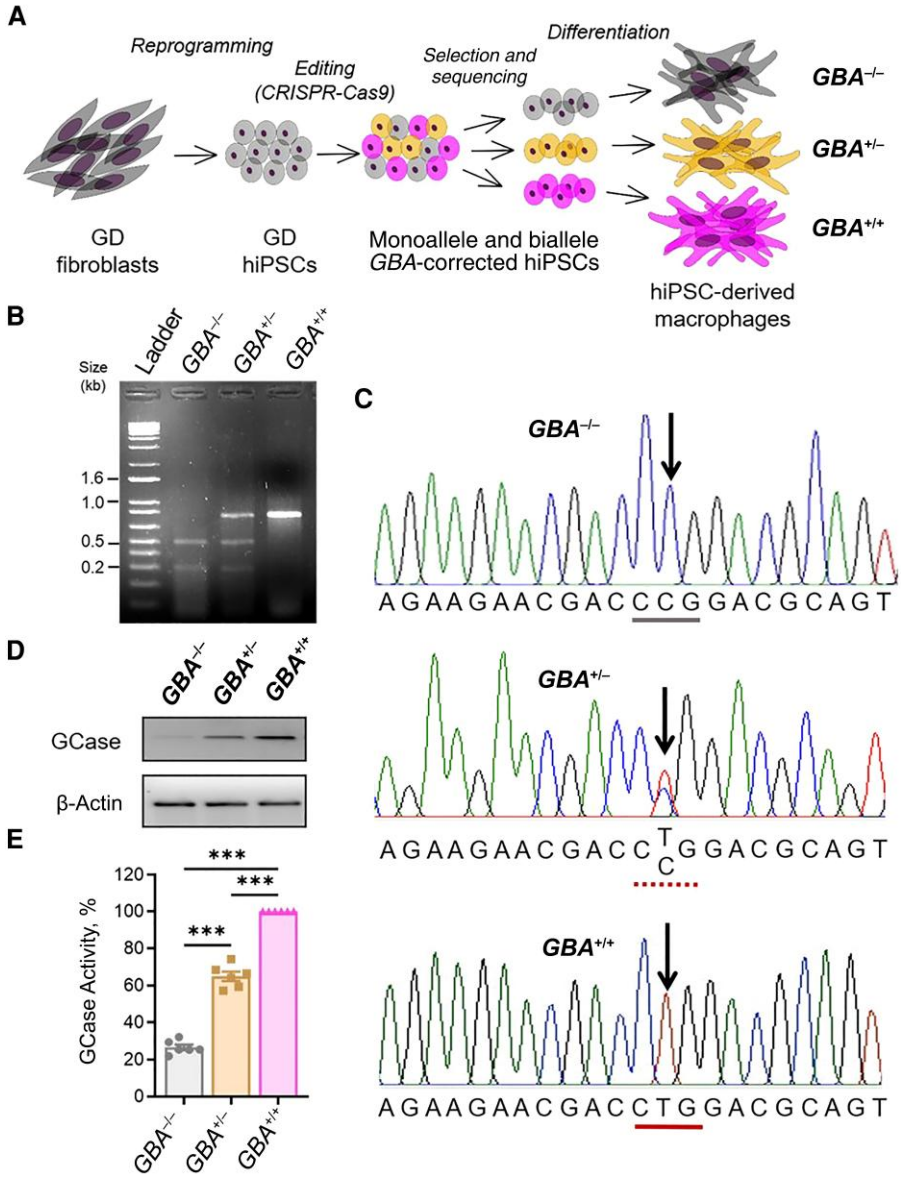

Figure 1. CRISPR (clustered regularly interspaced short palindromic repeats) correction of *GBA* mutations in Gaucher disease (GD) human-induced pluripotent stem cells (hiPSCs) restores normal β-glucocerebrosidase (GCase) activity to Gaucher macrophages. *A,* Schematic diagram showing the generation and genetic correction of GD hiPSCs and their differentiation into macrophages. *B,* Genotype characterization of $GBA^{-/-}$, $GBA^{+/-}$, and $GBA^{+/+}$ hiPSCs by *Nci*I restriction enzyme digest. *C,* Sequence profiles of the *GBA* mutation locus in $GBA^{-/-}$, $GBA^{+/-}$, and $GBA^{+/+}$ hiPSCs. *D,* Western blot analysis of GCase protein levels. *E,* GCase enzymatic activity relative to $GBA^{+/+}$ cells using 4-methylumbelliferyl β-D-glucopyranoside as the substrate: $GBA^{+/+} > GBA^{+/-} > GBA^{-/-}$(***$P < .001$).

with phagocytosis phenotypes, we observed that the migratory defect of $GBA^{-/-}$ macrophages was partially restored in $GBA^{+/-}$ macrophages, while biallelic *GBA* correction ($GBA^{+/+}$) fully restored cell motility compared with GD macrophages. Promisingly, our findings suggest that monoallelic L444P mutation correction may sufficiently enhance cell migration of Gaucher macrophages and thereby improve functional immune responses.

The isogenic hiPSC-derived GD and *GBA*-corrected macrophages offer an excellent model to investigate whether lysosomal disorders drive susceptibility to *M. tuberculosis* and whether *GBA* mutation correction restores normal lysosomal functions, *M. tuberculosis* susceptibility, and infectivity to Gaucher macrophages. *M. tuberculosis* replicates in macrophages by inhibiting phagosome-lysosome fusion. Lysosomal dysfunction might prevent the formation of tuberculosis granulomas which undergo secondary necrosis and thereby lead to altered tuberculosis susceptibility in patients with GD. To determine how GD and *GBA* mutation correction affect infectivity and growth of *M. tuberculosis* in macrophages, we

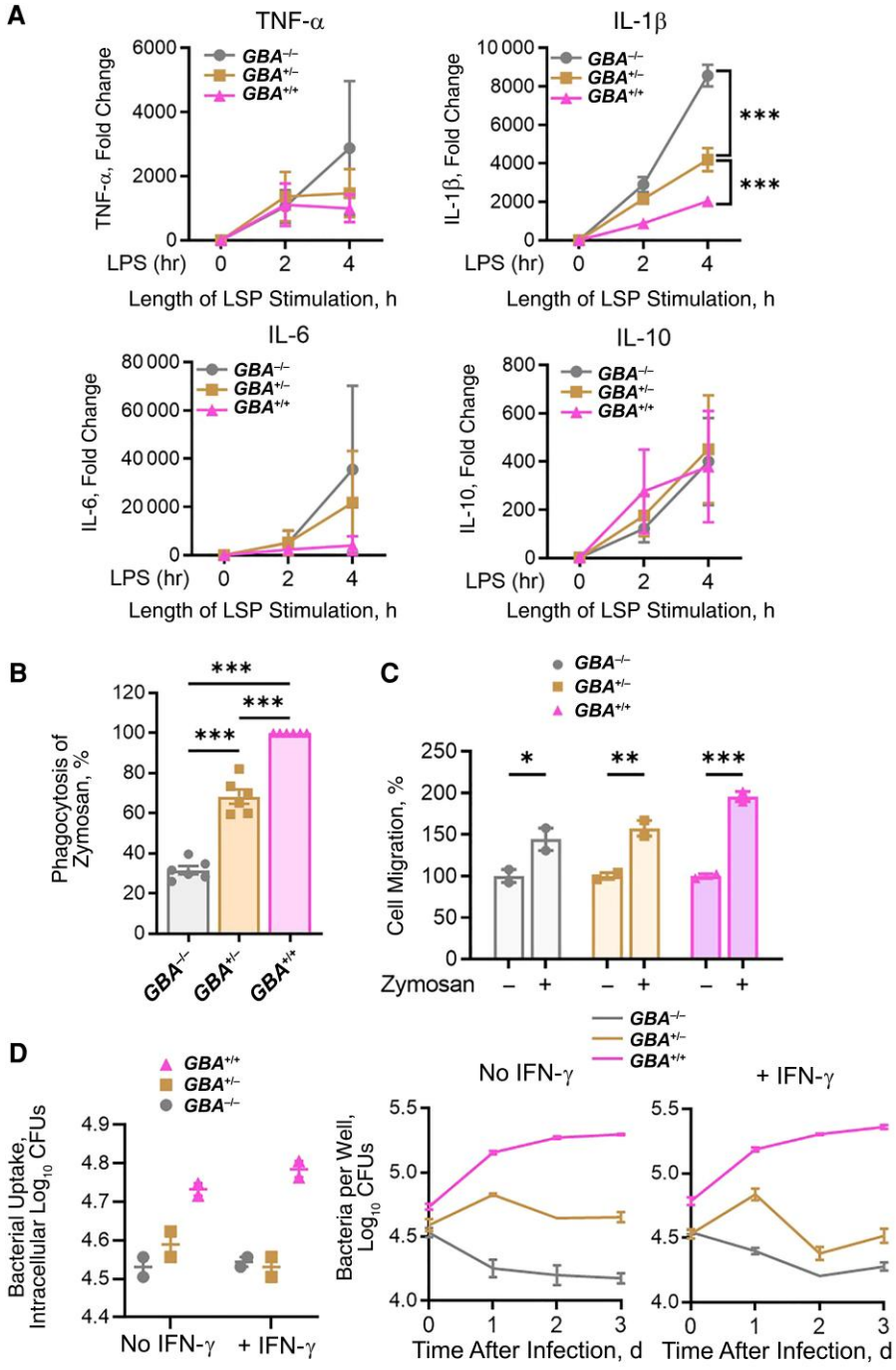

Figure 2. CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) correction of the *GBA* mutation in Gaucher disease (GD) human-induced pluripotent stem cells (hiPSCs) restores normal phagocytosis, motility, and immune functions to Gaucher macrophages and confers susceptibility to *Mycobacterium tuberculosis*. Functional properties of isogenic hiPSC-derived GD ($GBA^{-/-}$), monoallele ($GBA^{+/-}$), and biallele ($GBA^{+/+}$) *GBA*-corrected macrophages are compared. *A,* Tumor necrosis factor (TNF) α and interleukin 6, 1β and 10 (IL-6, IL-1β, and IL-10) transcript levels in isogenic $GBA^{-/-}$, $GBA^{+/-}$ and $GBA^{+/+}$ macrophages stimulated with lipopolysaccharide (LPS) for up to 4 hours. Significantly higher levels of IL-1β expression were observed in Gaucher macrophages (homozygous [$GBA^{-/-}$]and heterozygous [$GBA^{+/-}$]) than in biallele ($GBA^{+/+}$) *GBA*-corrected macrophages. *B,* Phagocytosis activity of macrophages using zymosan particles (50:1 exposure ratio for 30 minutes) relative to phagocytosis in $GBA^{+/+}$ macrophages. *C,* Analysis of cell motility by macrophage transwell migration assay. *D,* Analysis of macrophage susceptibility to *Mycobacterium tuberculosis* strain H37Rv by infection assays with an multiplicity of infection of 5:1 and an infection time of 4 hours. Graphs show bacterial uptake (*left*) and intracellular bacterial burden after infection of resting (*middle*; no interferon [IFN] γ) or activated (right; +IFN-γ) macrophages. The left panel represents the t = 0 intracellular $\log_{10}$ colony-forming unit (CFU) values shown in the middle and right panels. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

infected the isogenic $GBA^{-/-}$, $GBA^{+/-}$, and $GBA^{+/+}$ macrophages with *M. tuberculosis* H37Rv under biosafety level 3 conditions and monitored the intracellular bacterial burden within these cells (Figure 2*D*). Surprisingly, the cellular environment of GD macrophages impaired both the uptake and intracellular replication of H37Rv. In contrast, biallelic *GBA*-corrected macrophages supported robust H37Rv infection and growth, while bacterial replication was static in $GBA^{+/-}$ macrophages. Similar results were obtained for both unactivated and interferon γ–activated macrophages (Figure 2*D*).

GD macrophage membrane abnormalities are well established in the published literature [12]. Membrane abnormalities will vary depending on how well the GCase enzyme in GD macrophages degrades primary (GluCer) and secondary (GluSph) substrates since these substrates adhere to membranes disrupting the membrane properties [12]. This has the potential to decrease macrophage phagocytosis, as well as the availability of surface receptors for receptor-mediated endocytosis of *M. tuberculosis*. While our results show modestly reduced *M. tuberculosis* uptake by $GBA^{-/-}$ macrophages, their ability to inhibit bacterial proliferation was the major driver leading to the lower CFU count (approximately 1.0 $\log_{10}$ CFUs lower) at day 3 compared with $GBA^{+/+}$ macrophages, as shown in Figure 2*D*. Our findings suggest that GD may confer a level of protection against tuberculosis and that the *GBA* mutation correction increases Gaucher macrophage susceptibility to *M. tuberculosis*.

DISCUSSION

These findings lend credibility to the hypothesis that $GBA^{-/-}$ homozygosity and $GBA^{+/-}$ heterozygosity are protective against tuberculosis, and this may account in part for the persistence of GD mutations in human populations. Recently, Fan et al [12] showed that GD ($GBA^{-/-}$) zebrafish are resistant to infection with either *M. tuberculosis* or *Mycobacterium marinum* (a closely related mycobacterial species that is a natural fish pathogen) [13]. They further showed that this resistance is mediated by direct anti-bacterial activity by lysosomal GluSph which accumulates in *GBA*-deficient macrophages to high levels. GluSph is consistently elevated in GD macrophage lysosomes, and its toxicity was shown to have direct impact on mycobacterial survival [12]. The GluSph that accumulates in *GBA*-deficient macrophage lysosomes was both necessary and sufficient for increased mycobacterial killing [12]. Although pathogenic mycobacteria inhibit phagosome-lysosome fusion, *M. marinum*–containing phagolysosomes were nevertheless observed, and this was sufficient for a significant increase in the bactericidal activity of GD macrophages [12].

Interestingly, in contrast to our findings in which $GBA^{+/-}$ human macrophages showed intermediate resistance to *M. tuberculosis* infection, $GBA^{+/-}$ heterozygous zebrafish in the study by Fan et al [12] showed equivalent susceptibility to mycobacterial infection to that seen in wild-type fish. One possible explanation for this disparate observation is that, using zebrafish, Fan et al studied the N370S mutation, commonly observed in Ashkenazi Jews, which causes mild disease (nonneuropathic; type 1), modest life expectancy defects in homozygotes, and is associated with reduced but not abolished GCase enzymatic activity. In contrast, our work was done with hiPSCs carrying the L444P mutation which drastically reduces GCase activity and causes severe disease (neuropathic; type 2) in homozygotes and leads to death in early childhood.

In summary, we demonstrate that targeted CRISPR correction of a severe *GBA* mutation in hiPSCs restores normal functions to Gaucher macrophages. In addition, our findings support the hypothesis that GD confers protection against tuberculosis, consistent with a recent report using the zebrafish model [12]. Promisingly, our study suggests that it might be feasible to develop either hematopoietic (hematopoietic stem cell) or $CD34^+$ stem cell–based gene therapy as a permanent curative alternative to the expensive life-long GCase enzyme replacement therapy to treat nonneuropathic type 1 GD [14, 15].

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. W. R. B. and S. C. conceptualized the study. S. R., A. K., W. R. B., and S. C. designed the experiments. S. R. performed functional characterization of Gaucher disease human-induced pluripotent stem cells. H.M. performed CRISPR-associated protein 9 (Cas9) off-target cleavage analyses. A. K. performed macrophage phagocytosis, motility, and *Mycobacterium tuberculosis* infectivity assays. A. K. and S. K. performed data analyses and prepared the figures. W. R. B. and S. C. wrote the manuscript. D. N. R. provided advice and critical reading of the manuscript. W. R. B. and S. C. provided laboratory supervision. All authors reviewed and edited the manuscript.

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Potential conflicts of interest. All authors: No reported conflicts.

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consider relevant to the content of the manuscript have been disclosed.

References

1. Horowitz M, Wilder S, Horowitz Z, et al. The human glucocerebrosidase gene and pseudogene: structure and evolution. Genomics **1989**; 4:87–96.
2. Gupta N, Oppenheim IM, Kauvar EF, et al. Type 2 Gaucher disease: phenotypic variation and genotypic heterogeneity. Blood Cells Mol Dis **2011**; 46:75–84.
3. Panicker LM, Miller D, Awad O, et al. Gaucher IPSC-derived macrophages produce elevated levels of inflammatory mediators and serve as a new platform for therapeutic development. Stem Cells **2014**; 32:2338–49.
4. Panicker LM, Miller D, Park TS, et al. Induced pluripotent stem cell model recapitulates pathologic hallmarks of Gaucher disease. Proc Natl Acad Sci U S A **2012**; 109:18054–9.
5. Aflaki E, Stubblefield BK, Maniwang E, et al. Macrophage models of Gaucher disease for evaluating disease pathogenesis and candidate drugs. Sci Transl Med **2014**; 6:240–73.
6. Stirnemann J, Belmatoug N, Camou F, et al. A review of Gaucher disease pathophysiology, clinical presentation and treatments. Int J Mol Sci **2017**; 18:441.
7. Colombo R. Age estimate of the N370S mutation causing Gaucher disease in Ashkenazi Jews and European populations: a reappraisal of haplotype data. Am J Hum Genet **2000**; 66:692–7.
8. Diaz GA, Gelb BD, Risch N, et al. Gaucher disease: the origins of the Ashkenazi Jewish N370S and 84GG acid β-glucosidase mutations. Am J Hum Genet **2000**; 66: 1821–32.
9. Beutler E. Gaucher disease as a paradigm of current issues regarding single gene mutations of humans. Proc Natl Acad Sci U S A **1993**; 90:5384–90.
10. Kannai R, Elstein D, Weiler-Razell D, et al. The selective advantage of Gaucher's disease: TB or not TB? Isr J Med Sci **1994**; 30:911–2.
11. Ramalingam S, Annaluru N, Kandavelou K, et al. TALEN-mediated generation and genetic correction of disease-specific hiPSCs. Curr Gene Ther **2014**; 14: 461–72.
12. Fan J, Hale VL, Lelieveld LT, et al. Gaucher disease protects against tuberculosis. Proc Natl Acad Sci U S A **2023**; 120: e2217673120.
13. Berg RD, Levitte S, O'Sullivan MP, et al. Lysosomal disorders drive susceptibility to tuberculosis by compromising macrophage migration. Cell **2016**; 165:139–52.
14. Farfel-Becker T, Vitner EB, Futerman AH, et al. Animal models for Gaucher disease research. Dis Model Mech **2011**; 4:746–52.
15. Enquist IB, Nilsson E, Mansson JE, et al. Successful low-risk hematopoietic cell therapy in a mouse model of type 1 Gaucher disease. Stem Cells **2009**; 27:744–52.